AWARD NUMBER: W81XWH-13-1-0435

TITLE: Engineered Osteoclasts for the Treatment and Prevention of Heterotopic Ossification

PRINCIPAL INVESTIGATOR: Dr. Bruce Sangeorzan

CONTRACTING ORGANIZATION: University of Washington
Seattle, WA 98195

REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual	09/30/2014 - 09/29/2015

4. TITLE AND SUBTITLE
Engineered Osteoclasts for the Treatment and Prevention of Heterotopic Ossification

5a. CONTRACT NUMBER
W81XWH-13-1-0435

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. Bruce Sangeorzan, Dr. Cecilia Giachelli, Dr. Steven Bain, Dr. Susan Lund

Cameron Rementer, Dewayne Threet

E-Mail: mlund@uw.edu, bsangeor@uw.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Washington
4333 Brooklyn Ave NE
Seattle, WA 98195-0001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Heterotopic ossification (HO) is the abnormal formation of bone in soft tissues and is a frequent complication in patients who have suffered traumatic brain and spinal cord injuries. Currently there are few effective treatments for this condition. This research seeks to develop engineered osteoclasts as a local cell therapy for the prevention and/or regression of HO. The goals of this research are to develop proof-of-principle data in excised human HO specimens and in an animal model using existing murine engineered osteoclasts and to develop an off-the-shelf human cell source for clinical translation of this technology. To date we have performed studies to optimize the delivery and formation of engineered osteoclasts *in vivo*. In a pilot study, delivery and activation of engineered osteoclasts at the site of HO formation resulted in a reduction of HO formation. We have also validated a protocol for the production of human CD34+ cells from fibroblasts that we will combine with our CID technology for the production of human engineered osteoclasts.

15. SUBJECT TERMS
Heterotopic ossification, osteoclast, RANK, chemical inducer of dimerization, cell therapy

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	15	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

1. **INTRODUCTION:** Heterotopic ossification (HO) is the abnormal formation of bone in soft tissues and is a frequent complication in patients who have suffered traumatic brain and spinal cord injuries. Currently, there are few effective treatments for this condition. This research seeks to develop engineered osteoclasts as a local cell therapy for the prevention and/or regression of HO. The goals of this research are to develop proof-of-principle data in excised human HO specimens and in an animal model using existing murine engineered osteoclasts and to develop an off-the-shelf human cell source of engineered osteoclasts for clinical translation of this technology.

2. **KEYWORDS:** Heterotopic ossification, osteoclast, RANK, chemical inducer of dimerization, cell therapy

3. **OVERALL PROJECT SUMMARY:**

Current Objectives:

Major Task 1: To determine the ability of murine engineered osteoclasts to prevent or regress HO *in vivo* using a nude mouse model of HO generated by BMP2 injection.

- Subtask 1: Generation of RAW-iRANK cells for testing *in vivo* (PI: Giachelli, UW)
- Subtask 2: Optimization of local cell delivery at time of surgical implantation and after HO has formed in mice (PI: Sangeorzan, Harborview).
- Subtask 3: Optimization of CID dosing and route of administration to maximize osteoclast formation in mice (PI: Sangeorzan, Harborview)
- Subtask 4: Local delivery of engineered osteoclasts to prevent and treat HO formation following BMP2 treatment in mice.

Major Task 2: Test the ability of murine engineered osteoclasts to resorb calcified deposits in human HO samples derived from traumatic bone injuries.

- Subtask 1: Collection of human HO samples (PI: Sangeorzan, Harborview)
- Subtask 2: *In vitro* studies to assess the ability of engineered osteoclasts to resorb calcified deposits in human HO samples.
-

Major Task 3: To develop human iPS cells as a cell source for engineered osteoclasts.

- Subtask 1: Lentiviral construct, lentiviral production, and transduction of human iPS cells (PI: Giachelli, UW)

Results, Progress and Accomplishments with Discussion:

SOW Major Task 1: To determine the ability of murine engineered osteoclasts to prevent or regress HO *in vivo* using a nude mouse model of HO generated by BMP2 injection.

Subtasks 1-4: Optimization of local cell delivery and CID administration at the time of HO initiation and after HO has formed in mice. Local delivery of

engineered osteoclasts to prevent and treat HO formation following BMP2 treatment in mice.

In the past year, significant progress has been achieved both in the optimization of CID dosing to achieve osteoclast formation (Subtask 3) and the delivery of engineered osteoclasts to prevent HO formation (Subtask 4).

Experimentally, we first were able to demonstrate that delivery of RAW iRANK cells in a collagen carrier exerts a modest resorptive effect on BMP2-induced HO formation in Nu:J mice. For these experiments Nu:J mice were implanted with 1.0 mg/ml of BMP2 in a 10 ul of Matrigel in the mid-belly of the right calf to induce heterotopic bone formation. Following the BMP-Matrigel injection, the mice were allowed to ambulate freely for a period of 4 weeks. At the end of the 4 week period, mice underwent microCT scanning to confirm the development of HO lesions. Following the microCT scan, the mice were treated with RAW iRANK cells delivered via collagen or fibrin hydrogels. These two gel delivery vehicles were selected based on their ability to localize and retain engineered osteoclasts at the site of intramuscular injections in a previous pilot study. The collagen gel was formulated at two concentrations, 0.5 and 2.0 mg/ml, to determine the effects of hydrogel stiffness on osteoclast formation and function. The fibrin gel was only used at a concentration of 0.5 mg/ml, as higher concentrations of fibrin polymerized rapidly resulting in difficulty in the administration of the gel. Following cell delivery, CID was administered every two days for two weeks to activate osteoclast formation. The mice were scanned at Day 35 and Day 42 post-BMP2 injection to measure the HO lesion as well as tibia bone morphology. At the end of the 6 week study the animals were euthanized and the right calf muscle and tibiae were harvested and processed for histology.

As shown in Figure 1, delivery of engineered iRANK cells to the site of HO in collagen gels and subsequent activation with CID resulted in a decrease in HO volume over the two week study period. Further, the efficacy of the collagen gel carrier appeared to be superior to delivery of cells in fibrin hydrogels. Consequently, for subsequent *in vivo* experiments, collagen hydrogels were used as cell delivery vehicles.

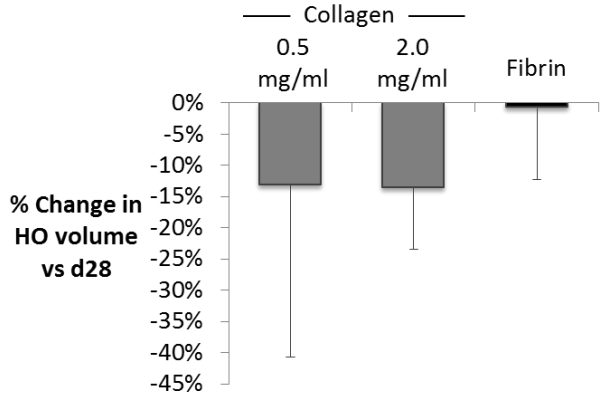

Figure 1. Mineralized HO volume in a murine HO treatment study. HO was induced in mice with BMP2 implantation and at Day 28 HO nodules were scanned by microCT. RAW iRANK cells were delivered to the site of HO formation in collagen (gray bars) or fibrin (black bar) hydrogels and engineered cells were activated with CID. HO volume was measured at the conclusion of the study at Day 42. Data presented as percentage change in mineralized HO volume at Day 42 vs Day 28.

Based on the encouraging results from the pilot study, we performed a second treatment study to elaborate on these initial findings. Additionally, as our *in vitro* data indicate that RAW iRANK cells are capable of secreting factors that actively inhibit calcification, we also included a prevention arm to determine if cell treatment at the time of HO induction could prevent the formation of HO. A further change to the study design was to administer multiple doses of engineered RAW iRANK cells. In the pilot study we administered a single dose of RAW iRANK cells, whereas in the current study we delivered RAW iRANK cells at multiple time points in an effort to maximize HO resorption.

For these experiments we performed a combination study with a 4-week prevention arm (n = 5 mice per group) and a 6-week treatment arm (n = 6 mice per group). In both arms, BMP2-Matrigel was implanted in the right calf to induce heterotopic bone formation. In the prevention study (Figure 2A) mice were treated with RAW iRANK cells delivered in collagen gels one-day post implant and again on Day 7, 14, and 21. Three control groups were also included; 1) untreated controls, 2) cells alone, 3) cells + CID. Following cell injections, CID was administered every other day to activate cells. Mice were scanned at Days 14 and 28 to measure HO lesions as well as tibia bone morphology. At the end of the 4-week prevention study, the mice were euthanized and the HO nodules, right calf muscle, and tibiae were harvested and processed for histology.

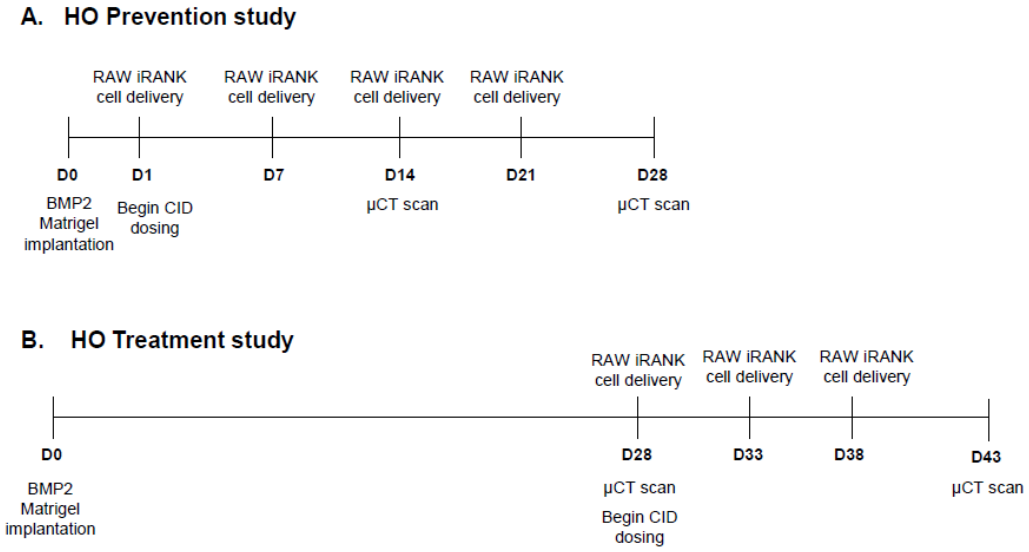

Figure 2. Timeline of HO prevention and HO treatment studies.

For the treatment arm of the study, the HO lesions were allowed to form for 4 weeks (Figure 2B). At the end of the 4 week induction period, HO lesions were localized by microCT and the mice were treated with RAW iRANK cells delivered in collagen gel carriers followed by CID administration. Additional cell treatments were scheduled for delivery on Days 33 and 38. CID was administered every two

days. At the end of the 2 week treatment study (Day 43) the final microCT scans were performed and the mice were euthanized and the HO nodules, right calf muscle, and tibiae were harvested and processed for histology.

As shown in Figure 3, results from the prevention study indicated that repeated injections of RAW iRANK cells delivered in collagen gel reduced the mineralized HO volume at Day 28 compared to Day 14. Mice treated with cells alone did not experience a reduction in HO volume from Day 14 to Day 28.

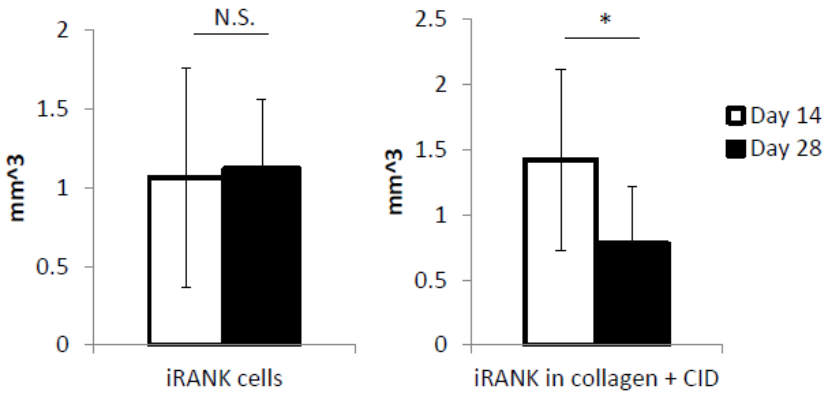

Figure 3. HO lesion bone volume in a murine HO prevention study. HO was induced in nude mice with BMP2 implantation. The next day RAW iRANK cells were delivered to the site of HO initiation and engineered cells were activated with CID. Cells were delivered again on Days 7, 14, and 21. HO volume was measured at Days 14 and 28. Data presented as mean ± SE. *p<0.05.

TRAP+ multinucleated cells were observed in tissues from mice that received cells in collagen gels + CID, demonstrating that engineered RAW iRANK cells can successfully form osteoclasts *in vivo* in response to CID treatment (Figure 4).

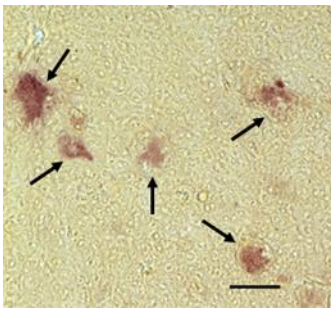

Figure 4. Engineered RAW iRANK cells form osteoclasts *in vivo* in response to CID treatment. TRAP positive multinucleated cells (indicated by arrows) were also positive for the iRANK GFP reporter (data not shown). Scale bar = 50 um.

In the prevention study, we observed that repeated cell injections resulted in an inflammatory/cell proliferative response in the hind limbs and we speculate that repeated intramuscular injections may have traumatized the soft tissue. We hypothesize that injecting a lower number of engineered RAW iRANK cells at each time point or performing fewer cell injections should minimize this problem. We are currently conducting experiments to optimize cell delivery parameters.

For the treatment study, repeated cell injections also led to inflammation in the calf muscle at the injection site. Consequently, the cell treatment scheduled for Day 38 was cancelled. At Day 37, HO lesions were measured by microCT and mice were euthanized. However, as shown in Figure 5, there was no change in mineralized HO volumes in mice treated with RAW iRANK cells in collagen gels.

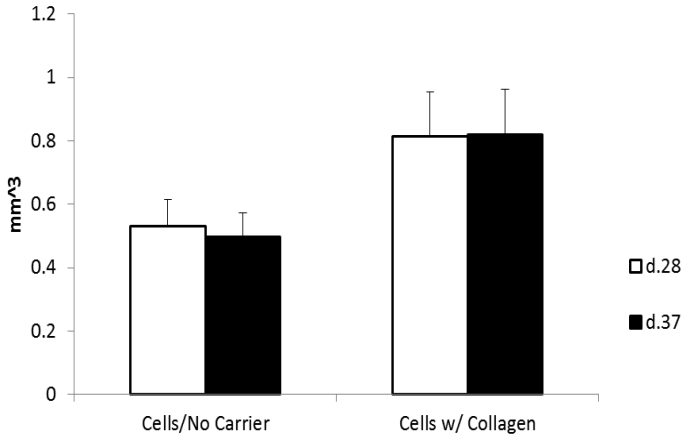

Figure 5. Mineralized HO volume in a murine HO treatment study. HO was induced in mice with BMP2 and at Day 28 HO nodules were scanned by microCT. RAW iRANK cells were delivered to the site of HO at multiple time points and engineered cells were activated with CID. HO volume was measured at the conclusion of the study on Day 37.

SOW Major Task 2: Test the ability of murine engineered osteoclasts to resorb calcified deposits in human HO samples derived from traumatic bone injuries.

Subtask 1: Collection of human HO samples (PI: Sangeorzan, Harborview)
This year human HO samples were collected from patients at the time of surgery. Human HO specimens were fixed in 70% ethanol and stored at -80°C for future use. As shown in Figure 6, sufficient material has been obtained to perform several experiments to optimize parameters for our *in vitro* osteoclast resorption studies.

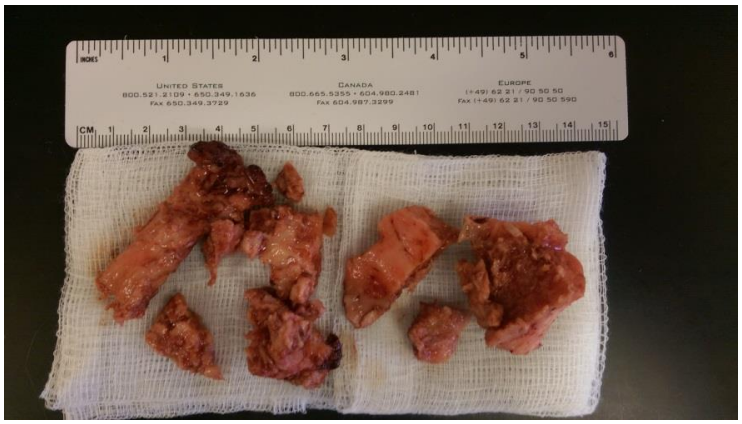

Figure 6. Human HO sample derived from patient with traumatic bone injury.

We have also begun experiments to determine the ability of our murine engineered osteoclasts to resorb calcification from these human HO specimens. For these studies, human HO samples were sectioned into 600 μm slices using a Buehler low speed saw equipped with a diamond wafering blade. Then a biopsy punch was used to create uniform 6 mm diameter discs (Figure 7). These were cleaned 2x by sonication in 70% ethanol for 5 minutes.

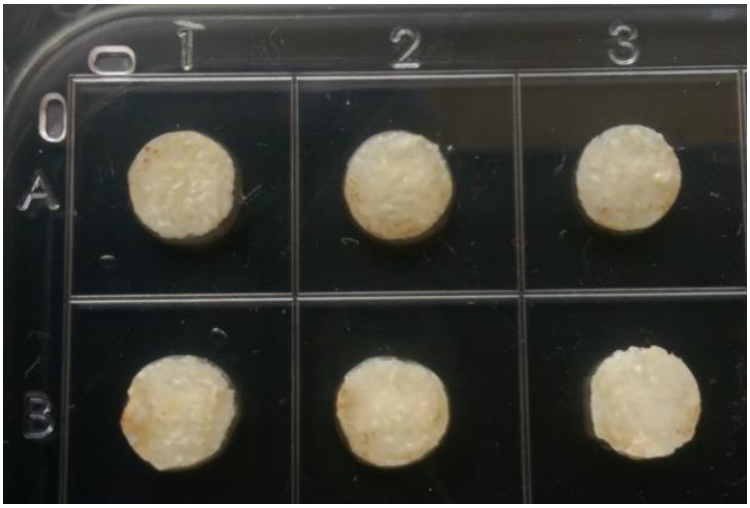

Figure 7. Human HO samples prepared for *in vitro* studies.

These discs were scanned by microCT to determine initial mineral volume and will be used for *in vitro* mineral resorption assays to evaluate our cell therapy.

- Optimization of engineered osteoclast formation *in vitro*: During this year we continued to refine our protocol for inducing osteoclast formation from RAW iRANK cells *in vitro.* We conducted a pilot study to determine the effect of media formulation on osteoclast formation. RAW iRANK cells were cultured in either DMEM or alpha-MEM for 5 days in the presence of CID. After 5 days, cells were fixed and stained for TRAP, and TRAP+ multinucleated cells were quantified. As shown in Figure 7, there was a trend towards increased osteoclast formation when RAW iRANK cells were cultured in alpha-MEM versus DMEM. We will use this protocol for osteoclast formation for our *in vitro* studies to determine the ability of our engineered osteoclasts to resorb mineral from human HO samples.

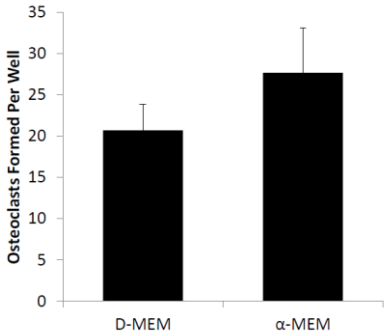

Figure 8. Effects of culture media formulation on osteoclast formation. RAW iRANK cells were cultured in either DMEM or alpha-MEM and treated with CID. Cells were then fixed and stained for TRAP.

SOW Major Task 3: To develop human iPS cells as a cell source for engineered osteoclasts

- <u>Optimization of osteoclast differentiation protocol for human iPS cells:</u>
  During the past year we have tested several protocols for the differentiation of human iPS cells to osteoclasts. Initially, we began with a co-culture protocol in which hiPSCs are cultured on OP9 stromal cells to induce hematopoietic differentiation. For these experiments, iPS(foreskin)-1 were plated on OP9 stromal cells to initiate hematopoietic differentiation. After 9 days of co-culture, cells were harvested and analyzed via flow cytometry for expression of CD43, CD235a, CD41, and CD45. Previously, we had experienced spontaneous adipogenesis of the OP9 stromal cells. When OP9 cells undergo spontaneous adipogenesis they no longer support hematopoietic differentiation. Consequently, we obtained a new lot of OP9 cells from ATCC. Unfortunately, this lot also underwent spontaneous adipogenesis. We also tested different formulations of media (powdered vs liquid), FBS sources (characterized vs. defined), and ascorbic acid concentration to determine the optimal parameters preventing spontaneous adipogenesis of OP9 and promoting hematopoietic differentiation of hiPSCs. However, we obtained low yield of $CD43^{+}CD235a/41^{-}CD45^{+}$ multipotent progenitor cells with all parameters tested.

- We next tested an embryoid body (EB) based method of hematopoietic differentiation for the production of osteoclasts from human iPSCs. This method involves sequential exposure of EBs to hematopoietic cytokines for the directed differentiation of hiPSCs (Figure 9).

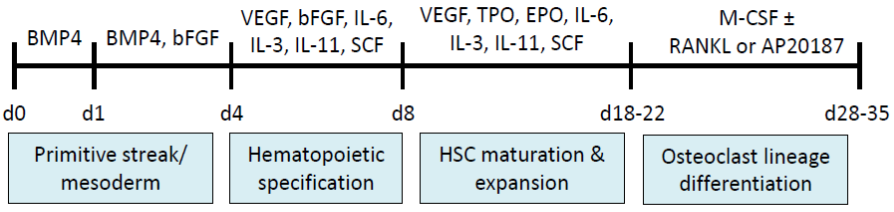

Figure 9. Stepwise protocol for hematopoietic differentiation of hiPSCs using an embryoid body based differentiation strategy.

- For these experiments EBs were generated from hiPS(foreskin)-1 cells and cultured in StemPro-34 with BMP4 for 24 hours. EBs were then harvested and cultured with BMP4 and bFGF to induce primitive streak/mesoderm formation. At day 4, EBs were harvested again and cultured with VEGF, bFGF, IL-6, IL-3, IL-11, and SCF to promote hematopoietic specification and development. At day 8, EBs cultured with VEGF, TPO, EPO, IL-6, IL-3, IL-11, and SCF for an additional 10 to 14 days for hematopoietic cell maturation and expansion. At this point EBs were dissociated with either trypsin or collagenase and myeloid precursors were cultured in the presence of M-CSF and RANKL for an additional 10 to 14 days to induce osteoclast formation. Cells were fixed and stained with TRAP to identify multinucleated TRAP-positive osteoclasts. However, we observed no TRAP+ or multinucleated cells with this method of osteoclast differentiation. EB dissociation method and length of culture periods were altered with no improvement in osteoclast induction.

- During this year we also began experiments to test a direct conversion protocol for hematopoietic differentiation that was recently published in *Stem Cells*. In this protocol, Sox2 overexpression in human fibroblasts is reported to induce the appearance of $CD34^+$ progenitor cells. For these experiments, we created a pCDH:Sox2 lentiviral vector. This lentiviral expression cassette has a GFP reporter downstream of a "self-cleaving" T2A peptide that will allow for analysis of transduction efficiencies via analysis of GFP expression. pCDH:Sox2 lentivirus was generated by co-transfecting HEK293T packaging cells along with the lentiviral packaging plasmids (pSL3, pSL4, and pSL5). Lentivirus containing supernatant was collected at 48 hours post-transfection and viral supernatant was concentrated via ultracentrifugation. Virus titer was determined by transducing HEK293T cells with lentivirus and determining GFP expression via flow cytometry. High titer virus (2.36×10^7 TU/ml) was produced.

  We then performed experiments to test efficiency of the direct conversion protocol. For these experiments, HFF-1 human neonatal fibroblasts were transduced with pCDH:Sox2 lentivirus to induce direct conversion. One day post-transduction, cells were cultured with dedifferentiation media and on Day 8 post-transduction cells were analyzed by flow cytometry for expression of CD34. GFP was also analyzed as a measure of Sox2 lentivirus transduction efficiency.

  As shown in Figure 10, transduction with lentivirus resulted in transduction efficiencies of greater than 65% for both the pCDH control and the pCDH:Sox 2 lentiviral constructs.

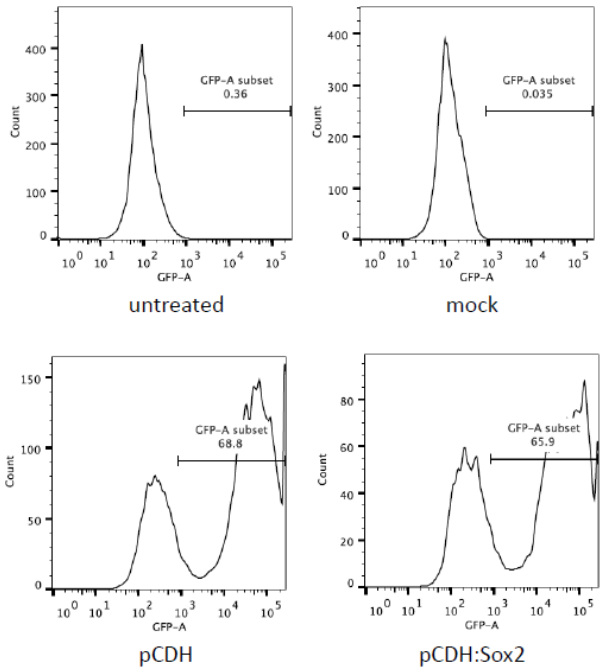

Figure 10. GFP expression in transduced HFF-1 fibroblasts. HFF-1 fibroblasts were transduced with pCDH lentivirus and GFP expression was analyzed by flow cytometry 8 days post-transduction.

CD34 expression was increased in HFF-1 fibroblasts transduced with pCDH:Sox2 lentivirus compared to fibroblasts transduced with the pCDH control lentivirus (Figure 11), indicating that Sox2 overexpression results the emergence of a CD34+ progenitor population.

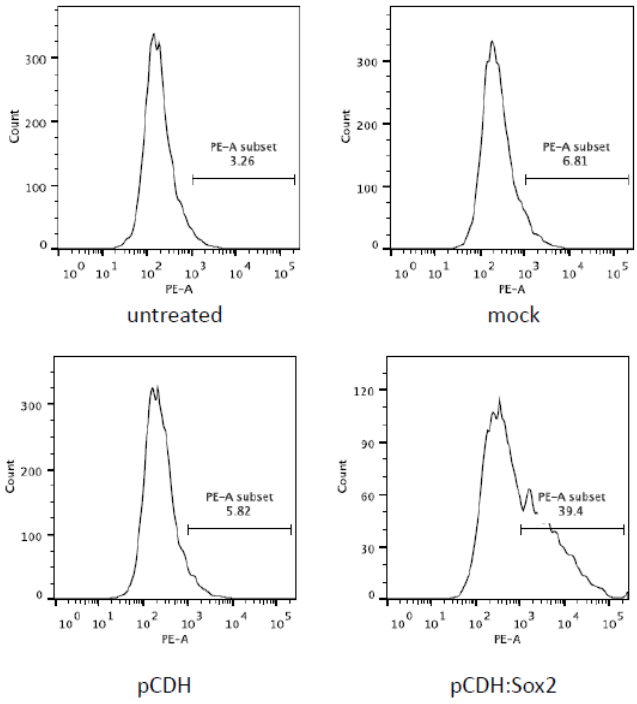

Figure 11. CD34 expression in transduced HFF-1 fibroblasts. Human HFF-1 cells were transduced with pCDH control or pCDH:Sox2 lentivirus. After 24 hours, media was switched to dedifferentiation media. On Day 8 post-transduction, CD34 expression was analyzed by flow cytometry.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated the formation of TRAP+ engineered osteoclasts *in vivo* in response to CID treatment (PI: Sangeorzan, Harborview)

- Conducted prevention study for the local delivery of engineered osteoclasts to the site of HO initiation. Data from the pilot study indicates that engineered osteoclasts can prevent HO formation *in vivo* (PI: Sangeorzan, Harborview)

- Collected human HO specimens for *in vitro* testing of our engineered osteoclasts (PI: Sangeorzan, Harborview).

- Generated CD34+ cells by direct conversion of fibroblasts via overexpression of Sox2 (PI: Giachelli, UW).

5. CONCLUSION:

Currently, few effective treatments exist to treat heterotopic ossification. Our studies will advance the field by determining whether osteoclasts can prevent and/or regress HO and provide insights into optimal conditions for the treatment of HO. The goals of this research are to develop proof-of-principle data in excised human HO samples and in an animal model using existing murine engineered osteoclasts and to develop an off-the-shelf human cell source for clinical translation of this technology.

Future Plans

SOW Major Task 1:

- Subtask 4: Local cell delivery of engineered osteoclasts to prevent and treat HO formation: Based on the progress and observations made in these studies, we are currently performing experiments to assess the viability and resorption capacity of a single injection of engineered osteoclasts into an ectopic bone nodule. In this study we will also be testing the efficacy of delivering purified osteoclasts to the ectopic bone nodule. Osteoclasts will be formed *in vitro* by culturing RAW iRANK cells with CID for 4-5 days. Osteoclasts will then be enriched by a serum gradient and injected. These experiments are currently in progress with initial results expected in December 2015.

SOW Major Task 2:

- *In vitro* studies to assess ability of engineered osteoclasts to resorb calcified deposits in human HO samples: With the human HO specimens we have collected and prepared we will perform *in vitro* experiments to determine the ability of our engineered osteoclasts to resorb mineral from human HO samples. For these experiments, RAW iRANK cells will be cultured on human HO specimens and resorption will be determined by microCT.

SOW Major Task 3:

- We will optimize parameters to differentiate CD34+ progenitor cells generated by direct conversion from fibroblasts further down the osteoclast differentiation pathway.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Abstracts:

Rementer CW, Ausk J, Gross TS, Bain SD, Sangeorzan BJ, Giachelli CM. *A Quantitative Method to Assess Engineered Osteoclastic Resorption of Heterotopic Ossification*. Abstract submitted to Vascular Biology 2015.

Lund, SA, Giachelli CM. *Development of Human Engineered Osteoclasts for the Treatment of Vascular Calcification.* Abstract submitted to Vascular Biology 2015.

7. INVENTIONS, PATENTS AND LICENSES: None to report.

8. REPORTABLE OUTCOMES:

- Demonstrated the formation of TRAP+ engineered osteoclasts *in vivo* in response to CID treatment (PI: Sangeorzan, Harborview)

- Conducted prevention study for the local delivery of engineered osteoclasts to the site of HO initiation. Data from the pilot study indicates that engineered osteoclasts can prevent HO formation *in vivo* (PI: Sangeorzan, Harborview)

- Collected human HO specimens for *in vitro* testing of our engineered osteoclasts (PI: Sangeorzan, Harborview).

- Generated CD34+ cells by direct conversion of fibroblasts via overexpression of Sox2 (PI: Giachelli, UW).

9. OTHER ACHIEVEMENTS:

None to report.

10. REFERENCES:

N/A

11. APPENDICES:

An updated quad chart is attached.

Engineered osteoclasts for the treatment and prevention of heterotopic ossification

OR120074P1

W81XWH-13-1-0435

PI: Dr. Bruce Sangeorzan **Org:** University of Washington, Orthopedics and Sports Medicine **Award Amount: $579,375**

Study/Product Aim(s)

- Aim 1: To determine the ability of murine engineered osteoclasts to prevent or regress HO in a nude mouse model.
- Aim 2: To test the ability of murine engineered osteoclasts to resorb calcified deposits in human HO samples.
- Aim 3: To develop human iPS cells as a cell source for engineered osteoclasts.

Approach

In this proposal, we will develop engineered osteoclasts to be used as a local cell therapy for the prevention and/or regression of HO. Our approach relies on a chemical inducer of dimerization to control osteoclast differentiation. The ability of murine engineered osteoclasts to resorb HO will be tested a) *in vivo* in a mouse HO model and b) *in vitro* using excised human HO specimens. We will also engineer human iPS cells and evaluate the engineered cells for osteoclast function.

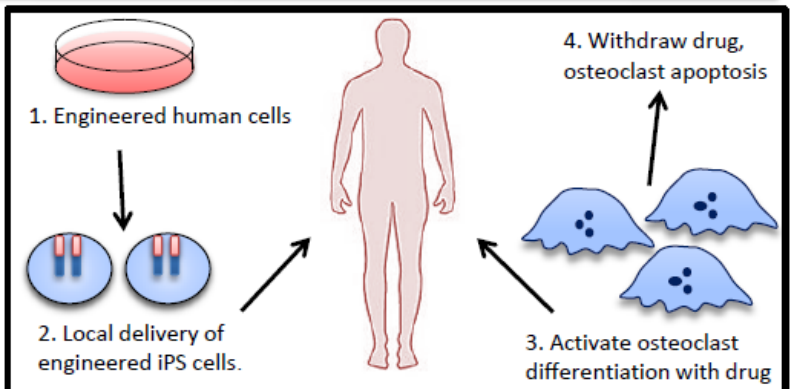

Figure 1: Off-the-shelf engineered therapy for HO

Accomplishment: Demonstrated formation of TRAP+ engineered osteoclasts *in vivo* in response to CID treatment. In a pilot study, engineered osteoclasts prevent HO formation *in vivo*.

Timeline and Cost

Activities / CY	13	14	15	16
Aim 1: Test engineered osteoclasts in HO mouse model				
Aim 2: Develop proof of principle data in excised human HO samples				
Aim 3: Develop engineered human iPS cell line				
Estimated Budget ($K)	**$48**	**$193**	**$193**	**$145**

Updated: (10/28/15)

Goals/Milestones

CY13 Goal

☑ Clone lentivirus construct for CID-inducible human RANK fusion gene

CY14 Goals

☐Optimize iPS transduction and CID-induced differentiation

☐Optimize local cell delivery and CID administration in mouse model.

CY15 Goal

☐ Assess engineered iPS cells for osteoclast function

CY16 Goal

☐ Test engineered osteoclasts in mouse HO model and human HO specimens

Comments/Challenges/Issues/Concerns

- None to report

Budget Expenditure to Date

Projected Expenditure: $377,157

Actual Expenditure: $356,250